Generation of Procoagulant Activity by Mononuclear Phagocytes: A Possible Mechanism Contributing to Blood Clotting Activation Within Malignant Tissues

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This study investigated the procoagulant activity (PCA) of mononuclear phagocytes from rabbits bearing the V2 carcinoma. Macrophages harvested from either intraperitoneally or subcutaneously growing tumors were found to express a very strong procoagulant activity as compared with peritoneal macrophages and circulating mononuclear cells from the same animals. On the other hand, when incubated with or without endotoxin in short-term cultures, circulating mononuclear cells from tumor-bearing animals generated significantly more procoagulant activity than those from control animals. In all instances, procoagulant activity was identified as tissue factor by using assay systems with plasmas selectively deficient in the various clotting factors. These results indicate that, besides cancer cells, mononuclear phagocytes might also play an important role in the activation of blood coagulation and in the deposition of fibrin at the host–tumor interface.

ACTIVATION OF BLOOD coagulation has been associated with malignancy in humans and laboratory animals. Fibrin deposition within and around tumors has been observed by several investigators, and malignancy is associated with a high incidence of vascular thrombosis and/or disseminated intravascular coagulation. It has been suggested that tumor procoagulants might be responsible for the activation of blood coagulation in malignancy. Mononuclear phagocytes are an integral part of the lymphoreticular infiltrate of experimental and human tumors, and neoplastic growth may be associated with profound changes in the functional state of mononuclear cells from different sites, such as altered phagocytes and enhanced prostaglandin generation in both unstimulated and stimulated cells.

In recent years, considerable evidence has accumulated that, upon exposure to different stimuli (endotoxin, immune complexes, complement proteolytic products, and others), mononuclear phagocytes generate a strong procoagulant activity that has been identified as tissue factor (TF). They are therefore capable of triggering blood coagulation through the extrinsic pathway.

In this study, we investigated the procoagulant activity of mononuclear phagocytes from rabbits bearing the V2 carcinoma. Tumor-infiltrating, peritoneal, and circulating mononuclear phagocytes were studied.

MATERIALS AND METHODS

The V2 carcinoma belongs to the Vx series of tumors derived from Shope virus papillomas of rabbit skin and has retained many morphological and functional features of a squamous cell carcinoma. After transplantation into outbred animals, the V2 carcinoma spreads in a reproducible manner, with vigorous local invasion, regular formation of regional lymph node metastases, and frequent lung metastases. After intraperitoneal inoculation, the tumor grows as a solid mass usually along the stomach, with concomitant formation of a macrophage-rich effusion. For the present experiments, male New Zealand rabbits were implanted with a V2 cell suspension either subcutaneously or intraperitoneally. All the experiments were performed 10–14 days after tumor implantation.

Peritoneal macrophages were prepared from rabbits bearing V2 carcinoma (implanted i.p.) as previously described. Briefly, mononuclear cells obtained by centrifugation on Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo, Norway) were incubated 45 min at 37°C in Eagle's Basal Medium on microexudate-coated Petri dishes (3003 Falcon, Oxford, CA). At the end of the incubation period, nonadherent cells were removed with jets of medium and macrophages were recovered by exposure for 5–10 min to 1 mM EDTA in phosphate-buffered saline. The cells were washed 3 times with Eagle's medium and finally resuspended in RPMI 1640 at the concentration of 0.5 × 10⁶/ml.

To isolate tumor-associated macrophages from solid tumors, specimens were minced mechanically and then exposed for 45 min to 0.3% collagenase (cat E0130, Sigma Chemical Co., St. Louis, MO, or Worthington Biochemical Corp., Freehold, NJ) in BME containing 10 μg/ml DNase. Disaggregated cells were centrifuged and resuspended in BME at a concentration of 2–5 × 10⁶ cells/ml. The cell suspension was seeded on microexudate-coated plastic and incubated for 30 min at 37°C with agitation every 5–10 min. Nonadherent cells were thoroughly washed off with jets of medium, and macrophages were recovered from plastic, washed, and resuspended at a concentration of 0.5 × 10⁶/ml, as described above. More than 90% of the peritoneal and tumor-associated adherent cells were mononuclear phagocytes as assessed by morphology, uptake of neutral red, and binding and phagocytosis of antibody-coated sheep red blood cells. The procoagulant activity of peritoneal and tumor-associated macrophages was determined immediately after preparation.
Mononuclear cell suspensions from whole blood of V2 carcinoma-bearing rabbits (5 implanted i.p. and 4 s.c.) and control animals were prepared after sedimentation of red cells with Dextran T-500 (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifugation of the resulting leukocyte-rich supernatant fluid on Ficoll-Hypaque according to Bühm. All preparations were washed 4 times with citrated phosphate-buffered saline [9 vol PBS and 1 vol 3.8% sodium citrate containing 0.5% (w/v) bovine albumin and 0.1% (w/v) glucose] and suspended in Hanks' balanced solution (Difco). Final cell suspensions contained more than 95% mononuclear cells. Monocytes defined by the criteria mentioned above comprised 18%-40% of the mononuclear cells in the preparations obtained from both tumor-bearing and control animals.

The ratio of platelets to leukocytes was always less than 0.5:1 as determined by light microscopy. Mononuclear cells from tumor-bearing rabbits were isolated simultaneously with those of control animals.

The capacity of peripheral blood mononuclear cells to produce PCA in response to endotoxin was studied as follows: each cell suspension from tumor-bearing and control animals was diluted with Hanks' solution to 0.5 x 10^6 monocytes/ml, mixed with endotoxin (1 μg/ml of Escherichia coli LPS W, Difco Laboratories), and incubated at 37°C for 6 hr. PCA was determined at the end of incubation.

Cell procoagulant activity was evaluated on both intact and disrupted (sonicated) cells by a one-stage clotting assay using autologous plasma as substrate. Clotting time was determined in duplicate in prewarmed plastic tubes, using the following test system: 0.1 ml cell suspension or buffer, 0.1 ml plasma, and 0.025 M CaCl2. In some experiments, human plasma from normal subjects or patients with congenital deficiency of factor X, VIII, or VII was used as substrate (Dade Inc., Pharmaseal, Trieste, Italy).

RESULTS

Rabbit macrophages harvested from solid V2 carcinoma were found to possess strong procoagulant activity, as measured by their capacity to shorten markedly the clotting time of autologous plasma (Fig. 1). Neither circulating monocytes nor peritoneal macrophages had appreciable PCA immediately after isolation (Fig. 1).

When incubated with and without endotoxin, peripheral blood mononuclear cells from V2 carcinoma-bearing rabbits generated significant higher procoagulant activity than mononuclear cells from control animals (means ± SE: 24 ± 2.4 versus 36.2 ± 3.3, p < 0.01 with endotoxin, and 50.9 ± 4.1 versus 80.9 ± 4.5, p < 0.02 without endotoxin; Student's t test for paired comparisons) (see Fig. 2). When tumor-associated peritoneal and endotoxin-stimulated mononuclear cells were disrupted by sonication before testing, results were similar (data not shown).

All cell types shortened the recalcification time of normal, factor VIII, or factor IX-deficient plasma to a similar extent, but had much less effect on factor VII-deficient plasma (Table 1).

Control experiments showed that incubation of normal mononuclear cells with collagenase or DNAase did not result in development of procoagulant activity (data not shown).

DISCUSSION

This study shows that the presence of a growing tumor, the V2 carcinoma, modifies the ability of host mononuclear cells to generate procoagulant activity in many respects. First of all, macrophages tested immediately after harvesting from the tumor mass, without any stimulation, expressed strong procoagulant activity. In contrast, peritoneal macrophages and circulating mononuclear cells tested immediately after isolation had no such activity. When incubated with and without endotoxin in short-term cultures, however, circulating mononuclear cells from tumor-bearing animals generated significantly more procoagulant activity than those from control animals. It should be mentioned that V2 carcinoma cells have a peculiar proco-

![Fig. 1](image-url)

**Fig. 1.** PCA of monocyte-macrophages from V2 carcinoma-bearing rabbits measured immediately after isolation. The concentration of mononuclear phagocytes in all cell suspensions tested was 0.5 x 10^6/ml. The clotting time of rabbit plasma recalcified in the absence of the cells (blank value) always exceeded 350 sec. Results are the mean ± SE of 4 separate experiments.

![Fig. 2](image-url)

**Fig. 2.** Generation of PCA by peripheral blood mononuclear cells from pairs of normal and V2-bearing rabbits. (A) PCA of mononuclear cells incubated in the absence of endotoxin. (B) PCA of endotoxin-stimulated cells. The height of the blocks represents the arithmetical mean of the results.
agulant activity, which is different from tissue factor in that it directly activates factor X.\textsuperscript{11,12} In all instances, the procoagulant activity was identified as a tissue factor. The mechanisms through which the presence of the tumor, in our experimental conditions, influenced the procoagulant activity of mononuclear phagocytes are not clearly understood. It is recognized that procoagulant activity may be induced in mononuclear cells by antigens, immune complexes, complement proteolytic products, endotoxin, or interaction with allogeneic cells in the mixed lymphocyte reaction.\textsuperscript{4} The host immune reaction to the tumor could therefore be responsible for the in vivo triggering of the procoagulant response in tumor-infiltrating macrophages. V2 tumor cells have been shown to contain different types of proteases,\textsuperscript{5} which might functionally alter macrophages, rendering them capable of generating procoagulant activity. Trypsin-induced activation of cellular tissue factor has been found in vitro by Maynard et al.\textsuperscript{13} The same V2 infiltrating macrophages were completely devoid of cytotoxic activity,\textsuperscript{14} offering further support to the concept that the procoagulant response cannot be corrected simply with other well known macrophage activities.

The exact pathophysiologic significance of the observed changes in procoagulant activity of mononuclear phagocytes is as yet unclear. Many investigators have reported activation of the blood clotting during the course of malignant disease, leading to fibrin deposition at the tumor site,\textsuperscript{1} and cancer cells have long been considered the main source of procoagulants responsible for this.\textsuperscript{1,2} The present results offer evidence that mononuclear phagocytes might also play an important role in the activation of blood clotting in association with malignant diseases.

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REFERENCES

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